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Rituximab induces clonal expansion of IgG memory B-cells in patients with inflammatory central nervous system demyelination

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ABSTRACT

Rituximab, a monoclonal B-cell cytolytic antibody, has beneficial effects in patients with inflammatory demyelinating diseases. So far, little data exists on B-cell subset recovery after rituximab treatment in inflammatory demyelinating diseases of the central nervous system (CNS). To elucidate whether rituximab promotes qualitative changes in the IgG memory B-cell repertoire we performed a single cell analysis in three patients with CNS demyelination. We did not observe any qualitative changes but detected an increased clonal expansion in the IgG memory B-cell compartment after treatment, indicating that a single course of rituximab does not eliminate specific IgG memory B-cells.

1. Introduction

Rituximab is a B-cell cytolytic chimeric IgG1 monoclonal antibody that targets the CD20 molecule, expressed on pre-B and mature B-cells, until their differentiation into plasma cells. Rituximab causes B-cell depletion by antibody-dependent cell-mediated cytotoxicity (ADCC), complement-mediated lysis and induction of apoptosis. Mainly peripheral B-cells are affected, whereas germinal-center B-cells and, in particular, marginal-zone B-cells were found to be resistant to killing (Edwards and Cambridge, 2006; Gong et al., 2005).

Rituximab has already been proven to have beneficial effects in patients with inflammatory demyelinating disorders (Bar-Or et al., 2008; Cree et al., 2005; Dalakas et al., 2009; Hauser et al., 2008; Kim et al., 2013; Kim et al., 2011; Monson et al., 2005), but so far little data exists on B cell subset recovery after rituximab treatment in inflammatory demyelinating diseases of the central nervous system (CNS). We previously reported that the therapeutic effect of rituximab in anti-myelin associated glycoprotein (MAG) neuropathy depends on efficient depletion of non-circulating B-cells and reconfiguration of the B-cell memory compartment (Maurer et al., 2012). In this study we could demonstrate that the long-term immunomodulatory effects of rituximab in anti-MAG neuropathy are mediated by the sustained reduction of expanded autoreactive IgM memory B-cells. Patients who responded to rituximab showed a reduction in the clonal expanded IgM memory B-cell compartment after therapy, which was associated with clinical disease remission. Interestingly, the frequency of clonal expanded IgG memory B-cells slightly increased after rituximab therapy. To prove whether this finding is unique to the anti-MAG-neuropathy patients, we additionally examined the peripheral IgG gene repertoire in patients with inflammatory demyelinating disorders of the CNS during rituximab therapy. We have analyzed one patient with aquaporin-4 (AQP4) antibody positive neuromyelitis optica (NMO), one patient with AQP4-antibody negative recurrent transverse myelitis and one patient with primary progressive multiple sclerosis (PPMS).

2. Materials and methods

2.1. Patients

The study was approved by the institutional review board of Medical University of Innsbruck (study no. UN3041 257/4.8) and all participants gave written informed consent. One patient with NMO (patient 1), one patient with recurrent myelitis (patient 2) and one patient with PPMS (patient 3) were included in this observational study (Table 1). Patients were treated with rituximab at a dosage of 375 mg/m^2 and 100–250 mg prednisolone was administered *i.v.* prior to each rituximab application. Patient 1 and patient 2 were recruited from a recently published study

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Table 1
Demographical, clinical and immunological characteristics of patients.

	Patient 1	Patient 2	Patient 3
Gender	Female	Male	Female
Age (Years)	20	21	46
Diagnosis	Neuromyelitis optica	Recurrent myelitis	Primary progressive multiple sclerosis
Disease duration before therapy (years)	4.3	0.6	5.0
Time from first to second sample (months)	5	7	9
Percentage of total B-cells before therapy	6.8	10.0	12.4
Percentage of IgG memory B cells before therapy	0.80	0.90	1.60
Percentage of total B-cells after therapy	0.7	2.0	0.6
Percentage of IgG memory B cells after therapy	0.05	0.10	0.05
AQP4-antibody titer before therapy	1:20,480	0	0
AQP4-antibody titer after therapy	1:20,480	0	0
Serum BAFF ng/ml before therapy	0.64	0.5	0.58
Serum BAFF ng/ml after therapy	3.45	5.11	2.55

on the effect of rituximab on patients with NMO spectrum disorders (NMOSD) from Medical University of Innsbruck (Gredler et al., 2013). Serum AQP4-antibody and B-cell activating factor (BAFF) levels were measured as previously described (Gredler et al., 2013; Mader et al., 2010).

2.2. Flow cytometry and single cell analysis

Single cell analysis was performed the same way as for the anti-MAG neuropathy patients as previously described (Maurer et al., 2012). In brief, frozen peripheral blood mononuclear cells (PBMC) were thawed in FACS buffer (2 mM EDTA, 5% FCS, 20 μg/ml DNAse [Roche] in PBS). After thawing, the PBMCs were strained (0.22 μm cell strainer; BD) and centrifuged for 15 min at 150 g and 4 °C. The cells were incubated with PE-conjugated anti-CD27 clone M-T271, PE-Cy7-conjugated anti-CD19 clone SJ25C1, biotin-conjugated anti-IgG clone G18-145, and FITC-conjugated streptavidin (all BD) and with component H (Invitrogen) for dead cell discrimination. Single $CD19^+$ $CD27^+$ IgG^+ memory B-cells were purified by flow cytometric cell sorting using a Facs Aria cell sorter and Diva software (both BD). Single cell sorting was directly into a 96-well plate (thermoquick PCR-Plate; Greiner bioOne) with 20 μl OneStep RT-PCR reaction mix (Qiagen) supplemented with 0.5 μM each of *Igh* variable region-specific forward and IgG reverse primers per well (Tiller et al., 2008). Directly after sorting plates were incubated for 60 min at 50 °C for reverse transcription. After the reverse transcription step, DNA polymerase was activated with an initial step of 15 min at 95 °C. Amplification of the resulting cDNA of *Igh* was performed at an annealing temperature of 58 °C for 45 s, an elongation temperature of 72 °C for 1 min, and a denaturation temperature of 94 °C for 30 s for 48 cycles. Nontemplate controls were included to exclude any contaminations in the master mix.

To reduce PCR error rates, we used the HotStar Taq DNA polymerase (Qiagen) with an error rate of 2×10^{-5} per nucleotide and cycle. PCR amplicons were separated by electrophoresis on a 1.2% agarose gel. To avoid cross contaminations on the gel, PCR products were physically separated from each other. Bands with the correct mass (approximately 500 bp) were excised using individual sterile scalpels; DNA was extracted (Qiagen MiniElute Gel Extraction Kit) and sequenced. The sequenced variable region of the amplified *Igh* was corrected with CLC Main Workbench and analyzed on the international immunogenetic information system (Brochet et al., 2008; Giudicelli et al., 2011). The variable region was analyzed for VH, DH, and JH usage; somatic hypermutations (SHM); CDR3 length; pI; and clonal expansion.

2.3. Sequence analysis

Clonal expansions were determined by similar CDR3 amino acid sequences and V_H, D_H, and J_H usage in the IgG memory B-cell pool. The length of the CDR3 was defined as the number of amino acids from the third position after the cysteine motive of the CDR3 to the tryptophan amino acid. For pI analysis, we used the entire amino acid sequence of the CDR3 and calculated it with the tool on ExPASy proteomics server (Gasteiger et al., 2003). The V_H, D_H, and J_H gene usage was weight balanced by the number of sequences derived from each donor to avoid tempering the result by different amount of sequences due to clonal expansion or PCR efficacy. For the SHM analysis, sequences were first blasted against the germline sequences in the database of IMGT.

2.4. Statistical analysis

The two-tailed Fisher exact test was used to compare frequencies of V_H, D_H and J_H gene family usage and the size of clonal expansions. All analyses are based on individual sequences, and data in figures, in which error bars are shown, are presented as mean ± SEM. Since we analyzed pooled sequence data, a repeated-observation analysis was not applied. A *P* value less than 0.05 was considered significant.

3. Results

3.1. No qualitative changes in the IgG gene repertoire after rituximab infusion

To determine whether rituximab induces qualitative changes in the peripheral IgG memory B-cell repertoire, we amplified and sequenced Ig heavy chain (*Igh*) genes of single sorted IgG memory B-cells ($CD19^+$ $CD27^+$ IgG^+) before and 4–9 months after therapy (Table 1). The percentage of the B-cell and the IgG memory B-cell pool within the peripheral lymphocyte compartment declined dramatically after rituximab therapy (Table 1). Nevertheless, we could sort sufficient B-cells for single cell analysis after therapy. Sequence analysis revealed no major differences in the V_H, D_H and J_H gene family usage between the patients (Fig. 1). Additionally, we could not find differences in the gene family usage of our patient cohort and the anti-MAG neuropathy patients and demographically matched healthy blood donors (Maurer et al., 2012). Furthermore, we could not find any significant differences in V_H, D_H and J_H gene usage before and after rituximab therapy (Fig. 1 and Supplementary Table). Next, we examined heavy chain characteristics of the complementary-determining region 3 (CDR3), as long and positively charged CDR3 regions have been associated with antibody-mediated autoreactivity (Wardemann et al., 2003; Yurasov et al., 2005). Again, we could not detect significant differences in length and isoelectric point (PI) after rituximab therapy in our patients. These data are compatible with our previously reported findings in rituximab-treated patients with anti-MAG neuropathy, at least for the IgG memory compartment (Maurer et al., 2012). We conclude that rituximab does not promote qualitative changes in the IgG memory compartment in patients with inflammatory demyelination.

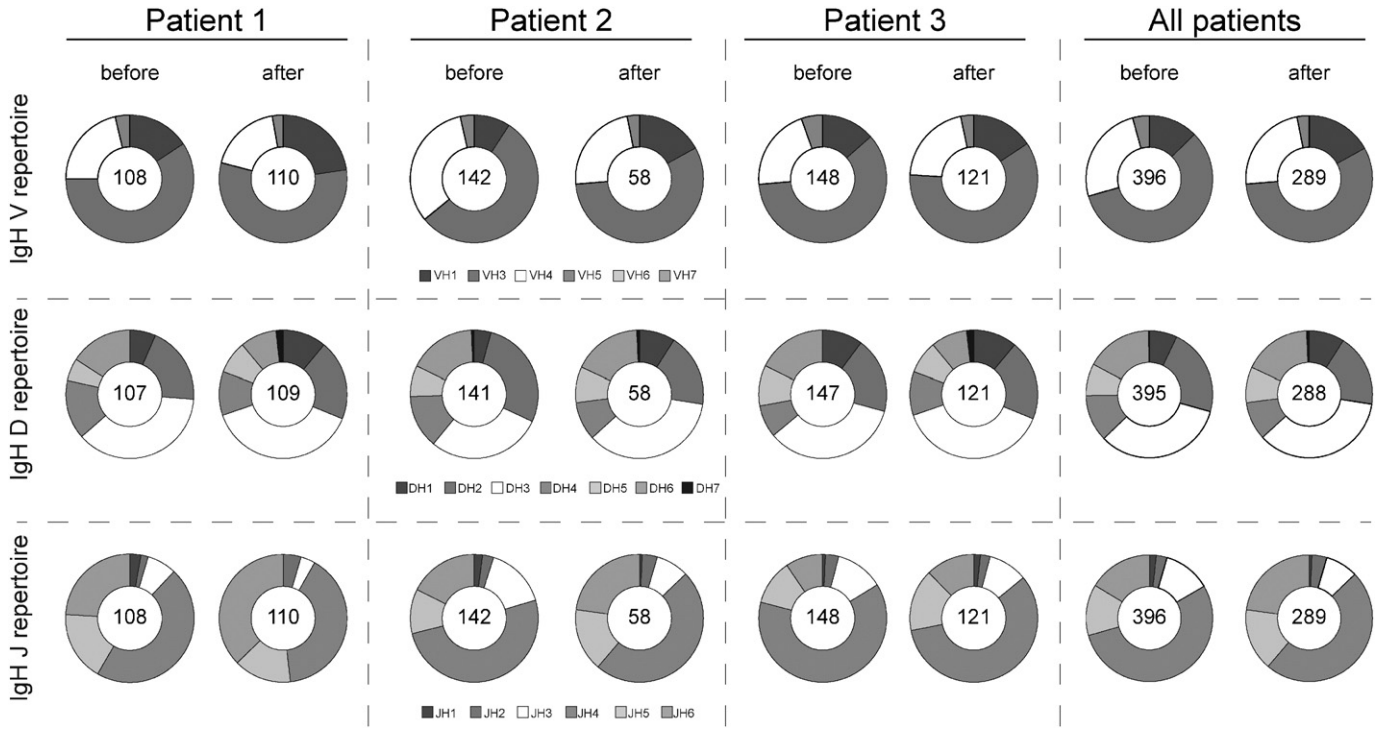

Fig. 1. IgG gene repertoire analysis in patients with NMO (patient 1), myelitis (patient 2) and MS (patient 3) and all patients before and 5–9 months after rituximab treatment. *Igh* gene variable (V) region, diversity (D) region and joining (J) region repertoires in single cell-sorted IgG memory B-cells ($CD19^+$ $CD27^+$ IgG^+) were analyzed. Clonally expanded sequences were counted as one sequence. Numbers within circles indicate the number of individual sequences analyzed per patient and time point. The 2-tailed Fisher exact test was used to evaluate V_H, D_H, and J_H repertoire.

3.2. Clonal expansion of IgG memory B-cells following rituximab therapy

Interestingly, the frequencies of clonally expanded IgG memory B-cells significantly changed in the three patients analyzed in this study before and after rituximab therapy. An increased IgG memory B-cell expansion was observed in all patients after rituximab therapy suggesting clonal expansion of non-depleted IgG memory B-cells, but not necessarily autoreactive ones (Fig. 2 and Supplementary Table). We could detect significantly more IgG memory B-cell expansions after therapy in all three patients ($P < 0.0001$), suggesting that the IgG memory B-cell pool was less efficiently depleted than the naïve B-cell compartment.

3.3. Increased serum BAFF levels after rituximab treatment

Additionally, we compared AQP4-antibody and serum BAFF levels shortly after treatment and during further follow-up and repeated

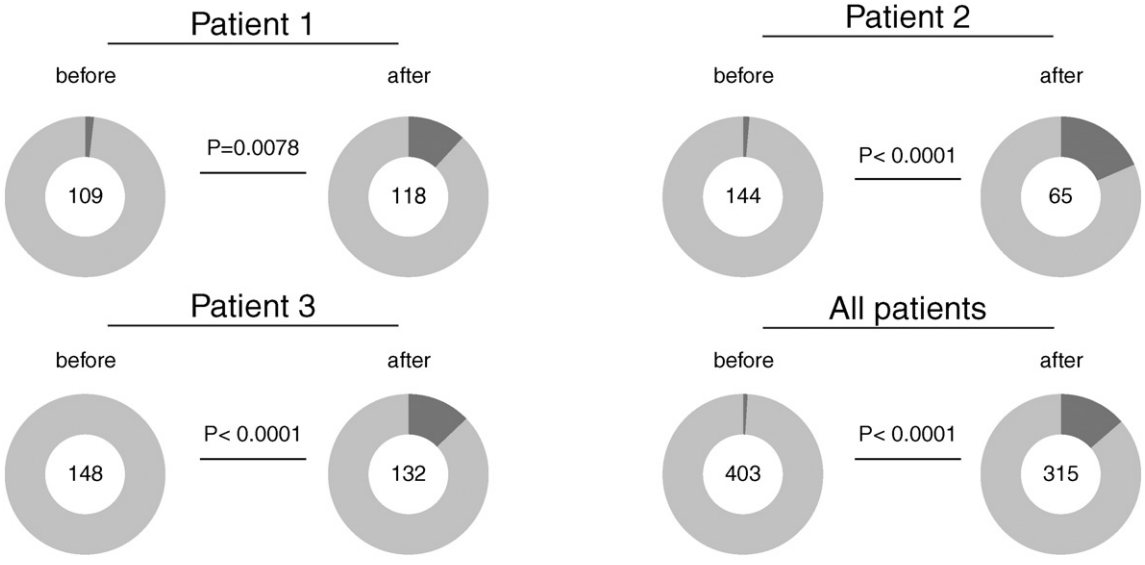

Fig. 2. Frequency of clonally expanded IgG memory B-cells patients with NMO (patient 1), myelitis (patient 2), MS (patient 3) and all patients 5–9 months after rituximab treatment. Numbers within circles indicate the number of individual sequences analyzed per patient before and after rituximab therapy. The frequency is represented as percentage of the total number of expanded sequences (dark gray pie area). This was calculated as a total number of expanded sequences including different expansions divided by the total number of the sequences. For example in the case of patient three one sequence was found two times before rituximab treatment of a total of 148 sequences and after treatment a total of 17 expanded sequences were found in 115 sequences.

rituximab infusions because previous studies suggested that repeated treatment with rituximab reduces AQP4-antibody levels and leads to an increase of serum BAFF levels (Gredler et al., 2013; Kim et al., 2013; Pellkofer et al., 2011). Antibody levels did not decline shortly after the first rituximab administration in our seropositive NMO case (patient 1), but we could observe a reduction in AQP4-IgG levels after long-term treatment as serum titer decreased from 1:20,480 to 1:640 after 1 year and to 1:160 after 3.5 years of repeated rituximab treatment. Serum BAFF levels dramatically increased after the initial treatment and stayed high during follow-up observations (Table 1).

4. Discussion

Our study demonstrates that a single course of rituximab does not promote any qualitative changes of the IgG memory B-cell repertoire but causes increased clonal expansion of IgG memory B-cells as well as increased serum BAFF levels. The effect of rituximab on the IgM memory B-cell repertoire in patients with IgM anti-MAG demyelinating neuropathy has already been investigated (Maurer et al., 2012). In accordance with this study, we report here the effect of rituximab on the peripheral IgG memory B-cell repertoire in patients with CNS autoimmunity, in which autoantibodies of the IgG class are more likely to affect the pathogenesis. The repopulated IgG clones do not show significant alterations in their V_H, D_H and J_H segment usage profiles. This was already demonstrated in the clonal expanded IgM repertoire of the anti-MAG patients. Repopulation and clonal expansion of the IgG memory compartment shortly after treatment contrasts with a previous study on depletion of panel reactive alloantibodies in which the naïve but not the IgG memory B-cells repopulate (Sidner et al., 2004). Similar results were recently obtained by Quan and colleagues who determined that after rituximab induced B-cell depletion, repopulation was characterized by the predominance of regulatory B-cells (Breg) rather than memory B-cells. Those Breg subsets are suspected to play a protective role in autoimmune diseases through production of Interleukin-10 (Quan et al., 2015). However, Leandro and colleagues could also find higher numbers of IgG memory B-cells in rheumatoid arthritis patients who relapsed on return of B-cells (Leandro et al., 2006). We conclude that a single dose of rituximab may not deplete the IgG memory B-cells efficiently, as the compartment expands shortly after administration. Whether the expanded IgG memory B-cells are autoreactive is still unknown since we did not test their antibodies reactivity. Nevertheless, we speculate that at least a few autoreactive cells could still survive, undergo clonal expansion and turn into short-lived antibody secreting plasma cells as serum antibody levels did not decline in our AQP4-antibody positive NMO patient after the initial rituximab infusion. Further investigation on single cell analysis in long-term treated patients could prove whether additional courses of rituximab lead to sufficient depletion of autoreactive IgG memory B-cells and will contribute to a better understanding on the impact of rituximab on peripheral IgG memory B-cells in autoimmune disorders. Moreover, it would be very important to see whether rituximab also affects the cerebrospinal fluid (CSF) B-cell pool because recent studies have indicated that the peripheral and CSF B-cell pools are only partly overlapping (Kowarik et al., 2015; Obermeier et al., 2008) and therefore it is unclear whether the phenomenon observed in our study might also occur in the CSF B-cell pool. Unfortunately this was not possible in our study because of the lack of CSF samples due to ethical reasons.

We could not observe an increase of AQP4-antibodies in our seropositive NMO patient after repopulation and expansion of the IgG memory B-cell compartment, which suggests that these cells are either not autoreactive, or they did not turn into antibody secreting plasma cells. On the contrary, we could observe a reduction of serum AQP4-antibodies after repeated rituximab infusion, suggesting that not only autoreactive B-cells, but also their corresponding plasma cells are, at least indirectly, affected. Previous studies on the long-term effect of rituximab on antibody titers of anamnestic antigens, such as tetanus toxoid, revealed that those titers remain stable, suggesting that rituximab has a different effect on autoreactive memory B-cells and their corresponding short-lived plasma cells, compared to certain anamnestic memory B-cells and their corresponding long-lived plasma cells, which are responsible for post-vaccination responses (Dalakas, 2008).

We further observed an increase in serum BAFF levels shortly after the first rituximab infusion. BAFF, a cytokine of the tumor necrosis factor family (TNF), is necessary for B-cell differentiation and survival. BAFF and it's corresponding TNF receptors were already proven to predispose to autoimmunity in experimental animal models (Gross et al., 2000; Mackay et al., 1999; Thompson et al., 2001). In human studies, levels of BAFF in the blood strongly increase during B-cell depletion and decrease when B-cells return (Edwards and Cambridge, 2006). Furthermore, increased serum BAFF levels were already associated with a higher risk of relapses shortly after treatment (Nakashima et al., 2011; Perumal et al., 2015). Interestingly, we could also observe a single relapse in our seropositive NMO case (patient 1) one month after the initial rituximab treatment that can be associated with increased serum BAFF levels. One possible explanation for this relapse might be that due to insufficient depletion, remaining B-cells undergo a fast clonal expansion upon increased BAFF signaling. Alternatively, reactivation of autoreactive long-lived plasma cells, which are not targeted by rituximab, leads to reactivation of disease. This observation is consistent with other reports on an increase in disease activity shortly after rituximab treatment (Nakashima et al., 2011; Perumal et al., 2015).

Our study has the following limitations: 1) the number of included patients is low and the three patients had different autoimmune demyelinating disorders. This selection was based on a recently published study on the effect of rituximab on patients with NMOSD (Gredler et al., 2013). We are aware that this heterogeneity might complicate interpretation of the results because they could be influenced by the underlying disorders. However, the larger number of patients with MS or NMOSD which have been treated with rituximab in clinical and case studies revealed a comparable beneficial effect of rituximab in these disorders (Bar-Or et al., 2008; Cree et al., 2005; Dalakas et al., 2009; Hauser et al., 2008; Kim et al., 2013; Kim et al., 2011; Monson et al., 2005). Further, our findings of increased clonal expansion of IgG memory B-cells are comparable with findings already reported in anti-MAG neuropathy patients (Maurer et al., 2012). In the anti-MAG study we analyzed the IgG memory compartment of 19 rituximab treated patients. The anti-MAG neuropathy patients showed a similar picture of the IgG memory compartment in regard to clonal expansions, VH and SHM as the three patients examined in this study (Maurer et al., 2012). Therefore we conclude that the three heterogeneous patients in this report show similar response to rituximab therapy as the 19 published patients with anti-MAG neuropathy. 2) Since the percentage of the IgG memory B-cells is very low after therapy (<2%) it is very likely that we find a sequence more then once. For an example if there is a small infection the memory B cell expands and newly formed IgG memory B-cell are less diluted in the total pool of B-cells. 3) The numbers of single memory B cells analyzed for VDL-segment usage was limited (up to 150 cells per patient) and thus it could be argued that they might not be representative of the IgG memory B cell compartment. However, similar numbers have been used in previous comparable studies (Amara et al., 2013; Benckert et al., 2011; Owens et al., 2007; Scheid et al., 2009; Tiller et al., 2007; Yurasov et al., 2006).

In conclusion, B-cell reconstitution following rituximab therapy is associated with clonal expansion of the IgG memory B-cell compartment but not with qualitative changes in the B-cell repertoire in the patients analyzed in this study. However, during long-term treatment AQP4-IgG serum levels decline, whereas serum BAFF levels remain high. Treatment with rituximab is a promising approach for immunotherapy of neurological diseases but as there is different contribution of B-cells in autoimmune disorders, and the degree of clinical response varies from patient to patient, further studies on autoreactive B-cells and their corresponding antibodies need to be performed.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2015.11.006.

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